

Copper oxide nanoparticles in clonal micropropagation of red oak

Olgae Zakharova^{1,2*}, *Alexander Gusev*^{1,2}, *Nataliya Strelakova*¹, *Gregory Grigoriev*¹, *Svetlana Chebotareva*¹, *Peter Baranchikov*¹ and *Anna Kataranova*¹

¹Scientific and educational center for Environmental Science and Biotechnology, Derzhavin Tambov State University, 392020 Tambov, Russia

²National University of Science and Technology «MISIS», 119991 Moscow, Russia

Abstract. Oak is an important tree species, playing a fundamental role in many forest ecosystems. Obtaining high-quality oak planting material is a actual issue in forest biotechnology. The most promising method for this, in vitro micropropagation, faces a number of problems that can be overcome using a nanobiotechnological approach. In our work, we obtained flaky copper oxide nanoparticles with a particle size of 50–200 nm in diameter and a thickness of 10–20 nm, which were used in the WPN medium at a concentration of 0.75, 1.5, 3, 6, and 15 $\mu\text{g L}^{-1}$ at the stage of introducing the original red oak material into the in vitro tissue culture. The study demonstrated a dose-dependent antimicrobial effect: seedling sterility increased from 80% (+10% to the control) at 1.5 $\mu\text{g L}^{-1}$ CuO to 100% at doses of 3 $\mu\text{g L}^{-1}$ and higher. The maximum survival rate was observed at 3 $\mu\text{g L}^{-1}$ – 43%, which is 23% higher than the control values. At the multiplication stage, nanoparticles significantly increased plant viability – twice as much in the variant with 3 $\mu\text{g L}^{-1}$ CuO and 1.7 times when using nanoparticles and phytohormones. The combined use of nanoparticles and hormones increased the seedling height by 1.5 times and the number of additional shoots by 3 times. At the rooting stage, CuO nanoparticles did not show any rhizogenesis-stimulating effect. At the same time, phytohormones and nanoparticles stimulated root formation. At the adaptation stage, a fairly low percentage of surviving and adapted plants was observed in the control variant, while the addition of nanoparticles had a positive effect on plant adaptation. The number of surviving seedlings increased by 15%, the number of adapted ones by 10. Thus, our study showed the prospects of using CuO nanoparticles to improve the biotechnology of clonal micropropagation of red oak. In the future, these results can be used in breeding and obtaining high-quality planting material for this species.

1 Introduction

Obtaining high-quality planting material for artificial reforestation and protective afforestation is a highly pressing issue. There is an undeniable need to search for new, effective, innovative technologies for accelerated production of healthy planting material of

* Corresponding author: olgazakharova1@mail.ru

tree species in vitro. Practical implementation of the concept of clonal afforestation can lead to the creation of more productive and genetically diverse forests [1]. It has been shown that the use of planting material obtained using clonal micropropagation techniques with the use of nanotechnological approaches can reduce production costs for afforestation and reforestation and make afforestation of territories more cost-effective [2]. The possibility of using nanoparticles in clonal propagation technology is dictated by their high antibacterial and antifungal activity [3-7], improved callusogenesis and organogenesis [8, 9], and the synthesis of biologically active compounds [10]. Among the most frequently used nanoparticles in plant growing, copper oxide nanoparticles can be distinguished, primarily due to their antimicrobial effectiveness, growth-promoting and protective properties [11, 12]. For example, CuO nanoparticles 20–50 nm in size exhibited strong antibacterial activity against gram-positive *Bacillus subtilis* and *Staphylococcus aureus*, gram-negative bacteria *Escherichia coli*, and against the fungi *Aspergillus flavus*, *Aspergillus niger* and *Penicillium frequentans* [13]. CuO nanoparticles obtained from the extract of *Zizyphus spina* leaves showed antifungal efficacy in vitro and in vivo against *Fusarium solani*, which causes root rot of tomatoes [14]. It was found on *Phytophthora nicotianae* that CuO nanoparticles affect the process of reproductive growth of the fungus, suppressing the growth of hyphae, spore germination and formation of sporangia [15]. Morphological damage, intracellular accumulation of ROS and increased activity of superoxide dismutase in hyphae are assumed as antifungal mechanisms. At the same time, activation of protective enzymes of tobacco and increased expression of resistance genes were observed.

In our previous works, the efficiency of CuO nanoparticles at different stages of clonal micropropagation of birch and grey poplar was demonstrated [16-18], and activity against phytopathogenic fungi *Alternaria alternata* and *Fusarium avenaceum* [19], *Alternaria solani* [20] was established. In the present study, the effect of CuO nanoparticles on red oak microseedlings at all stages of microcloning was assessed. Oak (*Quercus* L. spp.) is an important tree species, playing a fundamental role in many forest ecosystems. However, a number of threats, such as habitat loss and climate change, have led to a decline in the number of a number of species worldwide [21]. In addition, the most common strategy of seed storage after drying is not suitable for oak [22], since oak acorns do not tolerate drying [21, 23]. The use of biotechnology methods will allow preserving valuable selection objects that are difficult to propagate by traditional methods and obtaining healthy planting material. However, many experts note the difficulties in in vitro cloning of oak [24]. Particularly difficult is working with adult plant material; already at the initial stages of introducing segments of old trees into culture, there are difficulties in obtaining sterile morphogenic explants due to the presence of internal infection [25-27]. Also, oak is characterized by an unsatisfactory rooting phase [28], while this stage is fundamental for achieving the final goal - transplanting plants into non-sterile ex vitro conditions.

Previously obtained results [16-18] suggest that the introduction of copper oxide nanoparticles into cultivation media will help reduce plant infection, as well as increase the efficiency of clonal micropropagation technology of red oak.

2 Materials and methods

The study used copper oxide nanoparticles obtained by chemical precipitation [17]. The morphology of the obtained nanoparticles was studied by scanning electron microscopy on a Merlin high-resolution scanning electron microscope (Carl Zeiss, Germany).

To introduce the nanoparticles into the nutrient medium, an aqueous dispersion was prepared. A weighed portion of CuO powder (50 mg) was placed in a 100 ml glass container with a screw cap and filled with 50 ml of sterile distilled water (pH 7.1 ± 0.2). The suspensions were mixed and treated with ultrasound (VBS-41H, Vilitek, Moscow, Russia)

for 10 min. Aliquots were taken from the resulting concentrated suspension of nanoparticles (1 g/l) for introduction into the culture medium to obtain working concentrations. The dispersion composition and stability (measurement of zeta potential) of the initial colloidal solution were studied using a ZetasizerNanoZS analyzer (Malvern Panalytical, Malvern, UK).

The material for the study was red oak plants (*Quercus rubra*) growing in open ground conditions on the territory of the Semiluki forest nursery in the Voronezh region (Russia) (Fig. 1).



Fig. 1. Red oak in open ground.

Freshly cut cuttings with apical and axillary buds were used for the study. The cuttings were thoroughly washed using surfactants and rinsed with distilled water. After that, the shoots were cut into 3-5 cm pieces, washed in a solution containing 200 μ l of a 2% sodium hypochlorite solution and 200 ml of distilled water for 35 minutes, followed by rinsing in distilled water (at least 10 minutes), and then proceeding directly to sterilization. The main sterilization of the shoots was carried out for 15 minutes in a laminar flow hood in a solution containing 15 ml of a 5% sodium hypochlorite solution and 85 ml of sterile water. After the main treatment, the explants were subsequently rinsed 4 times with sterile water. For introduction into the culture, sterile explants were cut under aseptic conditions into 1.5-2 cm segments with one axillary bud and introduced into culture vessels with nutrient media containing CuO nanoparticles at concentrations of 0.75, 1.5, 3, 6, and 15 μ g/l. Cultivation took place on WPM (woody plant medium) [29] on shelves of the light installation of the culture room at a temperature of +24 °C, day/night photoperiod of 16/8 hours, illumination of 5000 lux, and relative air humidity of 70%.

WPM medium was also used at the multiplication stage. Cultivation was carried out in media containing hormones (0.2 mg/l benzylaminopurine (BAP), 0.1 mg/l indolyl-3-acetic acid (IAA), and 0.3 mg/l gibberellic acid (GA)), nanoparticles (3 μ g/l), and nanoparticles and hormones together.

During cultivation, morphometric parameters, seedling sterility, and condition were assessed on a 5-point scale:

- 5 points – ideal condition of microclones, green color, no necrotic foci;
- 4 points – good condition of microclones, green color, necrotic foci occupy no more than 10% of green mass;
- 3 points – satisfactory condition of microclones, necrotic foci occupy no more than 30% of green mass;

2 points – poor condition of microclones, necrotic or vitrified foci occupy more than 30% of green mass, such microclones have little chance of survival;

1 point – very poor condition of microclones, necrotic foci occupy more than 60% of green mass.

At the multiplication stage, a histological analysis of leaf blades was also performed using the VideoTesT-Morphology 4.0 hardware and software package.

At the rooting stage, $\frac{1}{2}$ WPM containing CuO nanoparticles (3 $\mu\text{g/l}$) and indole-3-butyric acid at a concentration of 0.4 mg/l were used.

The effect of nanoparticles on oak seedlings when transferred to non-sterile greenhouse conditions was assessed under greenhouse conditions (humidity 85-90%, temperature 22-24 °C) in 0.5 l containers containing neutral peat and perlite in a 3:1 ratio. The pH of the aqueous extract from the peat substrate was 6.7. A broad-spectrum regulator and adaptogen 24-epibrassinolide (EBL) was used as a positive control [30, 31].

In all biological studies, there were 10 seedlings in each control and experimental group, the experiments were carried out in triplicate biological and analytical replicates.

Statistical data processing was performed using Microsoft Excel 2010 (Descriptive Statistics package) using one-way analysis of variance (ANOVA) at a 5% significance level.

3 Results and discussion

3.1 Nanoparticles and their suspensions

Morphological analysis of the obtained CuO nanoparticles showed that the sample consists of aggregates of flocculent particles, the size of individual particles is 50 - 200 nm in diameter and 10 - 20 nm in thickness. Energy-dispersive X-ray spectroscopy (EDXA) showed that the analyzed powder is copper oxide, without any impurities (Fig. 2).

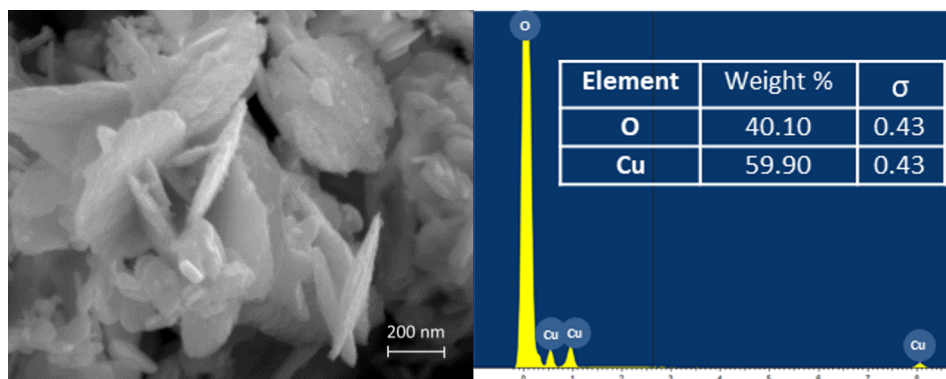


Fig. 2. Electron microscopic image and energy dispersive X-ray spectroscopy of a sample of CuO nanoparticles.

Analysis of the dispersed composition of the suspension showed that the average hydrodynamic diameter of particles and aggregates in the colloidal system was 150 - 250 nm (Fig. 3 a). The ζ potential of the original suspension of nanoparticles was - 36.6 mV (Fig. 3 b), which is evidence of the stability of the solution [32, 33].

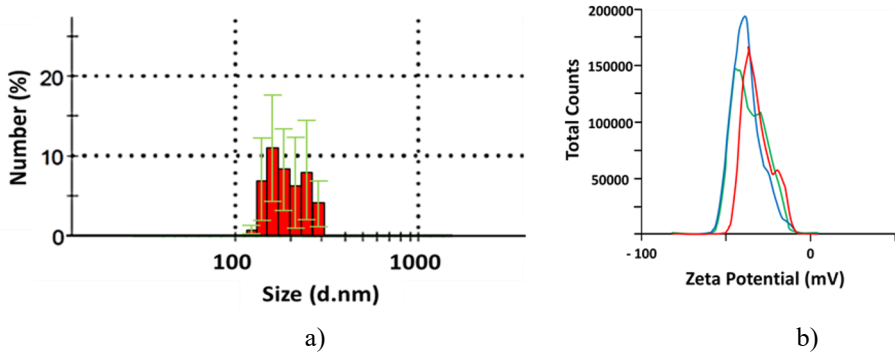
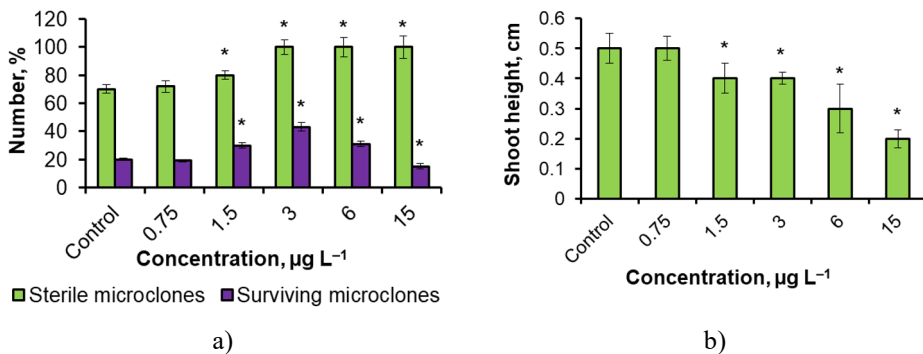


Fig. 3. The CuO-NP suspension (a) particle size distribution and (b) zeta potential.

The analysis of the obtained nanoparticle powder and their suspension showed that the obtained particles have a flocculent morphology, the maximum particle size is in the range of 50 - 200 nm with a thickness of 10-20 nm.

3.2 Microcloning

At the stage of introduction into the culture, concentrations of 0.75, 1.5, 3, 6, and 15 $\mu\text{g} / \text{l}$ CuO nanoparticles were used, introduced as a suspension (replacement of part of the water with a solution of nanoparticles) in the composition of the WPN nutrient medium. Cultivation was carried out for 1.5 months. During the study, it was found that the introduction of CuO nanoparticles into the cultivation medium had a dose-dependent antimicrobial effect (Fig. 4 a). Thus, at 1.5 $\mu\text{g/l}$, 80% of sterile shoots were noted, at 70% in the control variant. At higher doses (3...15 $\mu\text{g/l}$), 100% sterility was observed. The number of surviving seedlings in the control was about 20%, similar results were obtained for the 0.75 mg/l variant. The addition of 1.5 mg/l nanoparticles increased the percentage of surviving plants by 10%, and with an increase in concentration to 3 $\mu\text{g/l}$, the indicator increased by 23% relative to the control. With an increase in the concentration of nanoparticles to 6 $\mu\text{g/l}$, the percentage of surviving seedlings was 31% (+11% to the control), and at 15 $\mu\text{g/l}$, suppression of viability was already observed - the indicator was only 15%, which is 5% lower than the control values (Fig. 4 a). The height of plants decreased with increasing concentration of nanoparticles (Fig. 4 b), while the number of leaves was the same in all variants (2 pcs.), except for the group cultivated at 3 $\mu\text{g/l}$ of nanoparticles, where the average number of leaves was three (Fig. 4 c).



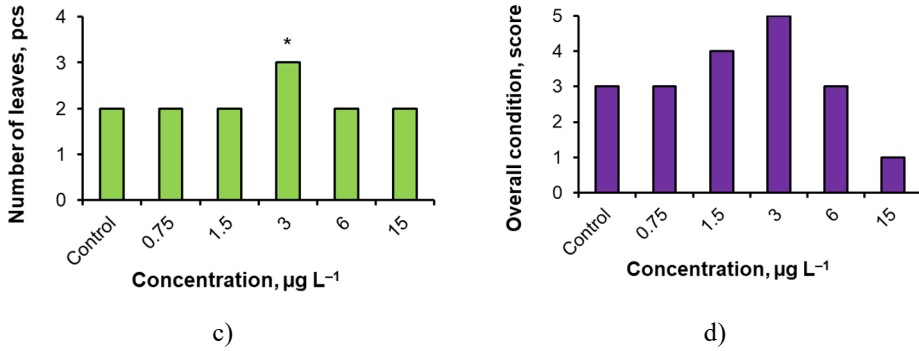
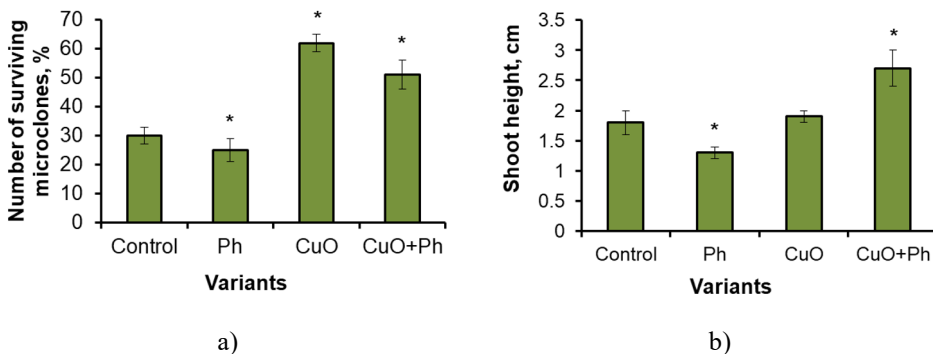


Fig. 4. Biomorphological parameters of red oak microclones at the stage of introduction into culture.

The assessment of the general condition of microclones is presented in Figure 4 d. The condition of seedlings was assessed at 5 points only in the 3 $\mu\text{g/l}$ group. In the control variant, the condition of plants was assessed at 3 points - small foci of necrotic damage were observed. At a concentration of 15 $\mu\text{g/l}$ of nanoparticles, strong inhibition of growth and development of seedlings was observed, necrotic foci occupied more than 60% of the green mass. The analysis of the obtained results shows that the most favorable conditions for plant cultivation at the stage of introduction into culture were those with a content of 3 mg/l of nanoparticles in the culture medium. This dose of nanoparticles was chosen for the further stage of multiplication. Cultivation was carried out on 4 variants of media: 1 - control, medium without hormones and nanoparticles, 2 - medium containing phytohormones (Ph), 3 - medium containing nanoparticles at a concentration of 3 $\mu\text{g/l}$ and 4 - medium with nanoparticles and hormones. At the stage of multiplication, copper oxide nanoparticles significantly increased the viability of plants (Fig. 5 a). In the control variant, the number of viable sprouts was only 30%, and the introduction of phytohormones further reduced the indicator by 5%. At the same time, in the group cultivated in a medium with nanoparticles, the number of surviving shoots was 62%, i.e. more than twice as high as the control values. With the combined use of nanoparticles and phytohormones, the number of surviving shoots (51%) was less than in the variant with only nanoparticles, but significantly more than with only phytohormones. The height of the seedlings did not change under the influence of nanoparticles (Fig. 5 b). Phytohormones in the nutrient medium reduced the indicator by almost 30%, but the combined use of nanoparticles and hormones allowed to increase the height of the seedlings by 1.5 times.



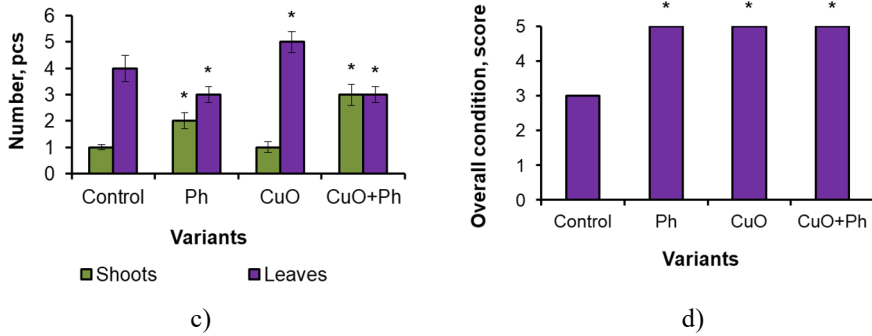


Fig. 5. Biomorphological parameters of red oak microclones at the multiplication stage.

The main objective of the multiplication stage is to increase the number of microclones. The addition of phytohormones to the culture medium increased the number of additional shoots by 2 times, and the use of phytohormones with nanoparticles increased the indicator by 3 times (Fig. 5 c). During the analysis of the experimental results, a decrease in the number of leaves on the seedlings under the influence of phytohormones was noted. The maximum number of leaves was observed in the plants of the group cultivated only with CuO nanoparticles - 5, against 4 in the control variant. Despite some decrease in a number of parameters in individual variants, the general condition of the seedlings in all experimental groups was estimated at 5 points, while the plants of the control group had foci of necrotic damage and their condition was estimated at 3 points.



Fig. 6. Red oak microclones: (left to right) control, CuO + hormones.

At the multiplication stage, a comparative histological analysis of the stomata of the leaves of the control group plants and plants grown with the addition of nanoparticles was carried out. As can be seen from Figure 7, in the CuO variant, a decrease in the area of the stomatal slit was observed, without a decrease in the density of the stomata compared to the control. This may indicate the resistance of these microclones to infectious diseases.

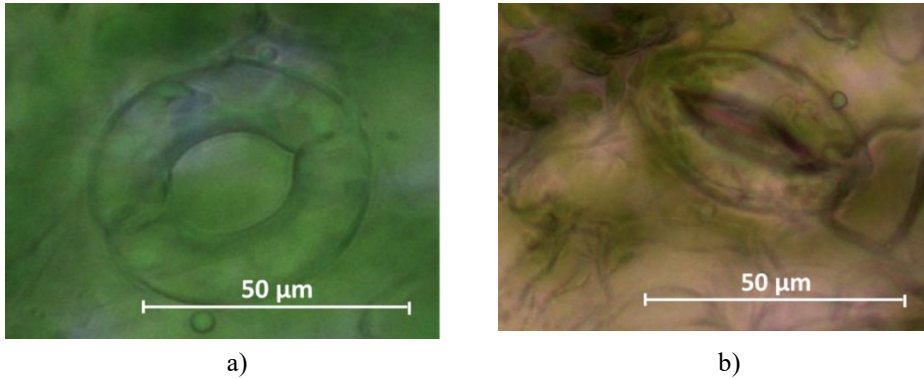


Fig. 7. Red oak stomata: a) control; b) CuO.

Thus, the studies showed the ability of CuO nanoparticles to increase the viability of red oak microclones by 32% compared to the control, the formation of adventitious shoots up to 2 pcs. (reproduction coefficient 3), increasing the adaptive capacity to in vitro conditions and infections of such a complex tree crop.

At the rooting stage, CuO nanoparticles did not show any rhizogenesis-stimulating effect (Table 1). However, when using phytohormones and nanoparticles, root formation occurred (Fig. 8).

Table 1. Morphometric indices of cultivated red oak plant samples at the rooting stage.

Variant	Number of surviving microclones, %	Number of microclones with roots, %
Control	100	0
CuO 3 g/l	100	0
IAA 0.4 mg/l	100	4
CuO + IAA	100	5



Fig. 8. Red oak microclones on the medium with IMC + CuO (left) and on the medium without additives (right).

In general, based on the results of the experiment, it can be said that copper oxide nanoparticles do not have a significant effect on the rhizogenesis of red oak seedlings.

At the stage of adaptation to non-sterile conditions, nanoparticle solutions at a concentration of 3 $\mu\text{g/l}$ were also used. It should be noted that the percentage of surviving and adapted plants in the control variant was quite low, which may be due to the difficulties in *in vitro* cloning of oak [24], while the addition of nanoparticles had a beneficial effect on plant adaptation (Fig. 9). The number of surviving seedlings increased by 15%, the number of adapted ones by 10. Analysis of the data shown in diagram 9 shows that the effect of nanoparticles is comparable to the effect of 24-epibrassinolide.

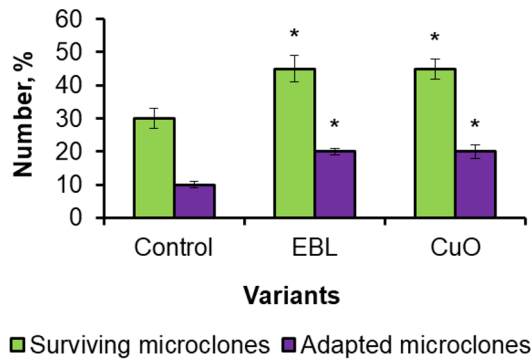


Fig. 9. Biomorphological parameters of red oak microclones at the stage of adaptation *in vivo*.

Thus, in our work, we obtained copper oxide nanoparticles of a flake shape, with a particle size of 50-200 nm in diameter and a thickness of 10-20 nm.

A dose-dependent antimicrobial effect was noted at the stage of introduction into the culture - the sterility of seedlings increased from 80% (+ 10% to the control) at 1.5 $\mu\text{g/l}$ CuO to 100% at doses of 3 $\mu\text{g/l}$ and higher. Copper-containing nanoparticles are known for their antimicrobial properties [34-36], in particular, a significant number of studies are devoted to the effect of copper nanoparticles on phytopathogens [37-40]. It is assumed that the main role in the biocidal mechanism is played by free copper ions released from the surface of nanoparticles and reactive oxygen species (ROS), causing oxidative stress [34]. Also, toxic effects described in bacteria and fungi include membrane damage [41], intracellular ion accumulation, protein inactivation, and DNA damage [42]. The maximum seedling survival rate in our study was observed at 3 $\mu\text{g/l}$ – 43%, which is 23% higher than the control values. Increasing the CuO concentration to 15 $\mu\text{g/l}$ decreased the rate by 5%, which may be due to the phytotoxic effect of copper [43-45]. Plant height decreased with increasing nanoparticle concentration, while the number of leaves was the same in all variants (2 pcs.), with the exception of the group cultivated at 3 $\mu\text{g/L}$ nanoparticles, where the average number of leaves was three. The general condition of the microclones was assessed at 5 points; the condition of the seedlings was assessed only in the 3 $\mu\text{g/l}$ group. In the control variant, the condition of the plants was assessed at 3 points, since foci of necrotic damage were observed. At a concentration of 15 $\mu\text{g/l}$ of nanoparticles, the condition was assessed at 1 point.

At the multiplication stage, nanoparticles significantly increased plant viability – twice as much in the variant with CuO and 1.7 times when using nanoparticles and phytohormones. Previously, other authors have shown that CuO nanoparticles improve plant growth and development by enhancing photosynthesis [46], nutrient absorption and root growth [47]. At the same time, in our experiment, the introduction of only phytohormones reduced the indicator by 5%. The height of seedlings did not change under the influence of nanoparticles, phytohormones reduced the indicator by almost 30%, but the combined use of nanoparticles and hormones allowed us to increase the height of seedlings by 1.5 times. The addition of phytohormones to the culture medium increased the number of additional shoots by 2 times,

and the use of phytohormones with nanoparticles increased the indicator by 3 times. A decrease in the number of leaves on seedlings under the influence of phytohormones was also noted. The maximum number of leaves was observed in the plants of the group cultivated only with CuO nanoparticles – 5, against 4 in the control variant. Despite some decrease in a number of indicators in individual variants, the general condition of seedlings in all experimental groups was estimated at 5 points, while plants of the control group had foci of necrotic damage and their condition was estimated at 3 points. Histological analysis of stomata of leaves of plants of the control group and plants grown with the addition of nanoparticles showed a decrease in the area of the stomatal slit in the variant with CuO, without a decrease in the density of stomata compared to the control. This may be evidence of the resistance of these microclones to infectious diseases. It is known that plants close their stomata upon detection of a molecular pattern associated with a pathogen to prevent the penetration of pathogens, which is known as stomatal immunity [48]. A complex regulatory network modulating stomatal defense against pathogens may include the perception of pathogen-associated molecular patterns, the production of nitric oxide and reactive oxygen species, calcium influx, activation of ion channels, and crosstalk between abscisic acid and jasmonic acid hormones [49, 50].

At the rooting stage, CuO nanoparticles did not show a rhizogenesis-stimulating effect. However, with the combined use of phytohormones and nanoparticles, as well as with phytohormones alone, root formation was observed.

At the adaptation stage, a fairly low percentage of surviving and adapted plants was observed in the control variant, while the addition of nanoparticles had a beneficial effect on plant adaptation. The number of surviving seedlings increased by 15%, the number of adapted ones by 10.

4 Conclusions

Thus, our study showed the potential of using CuO nanoparticles to improve the biotechnology of clonal micropropagation of red oak. In the future, these results can be used in selection and obtaining high-quality planting material for this crop.

5 Funding

Works were funded by the Russian Science Foundation, grant number 24-16-20039.

References

1. D. G. Thompson, *Clonal Reforestation: Forests of the Future?* In Seedling Physiology and Reforestation Success: Proceedings of the Physiology Working Group Technical Session (Springer Netherlands, Dordrecht, 1984)
2. M. Sijacic-Nikolic, A. Ivanova, N. Sirotkina, IOP Conf. Ser: Earth Environ Sci., **595**, 1 (2020)
3. T. Grodetskaya, *Cuo Nanoparticles in Clonal Micropropagation of Woody Plants*. In Proceedings of the VII International Scientific Conference Plant Genetics, Genomics, Bioinformatics and Biotechnology, (PlantGen) 2024, Kazan, Russia (2024)
4. A.R. Cruz-Luna, H. Cruz-Martínez, J Fungi (Basel) **7**, 12 (2021)
5. S.A. Al-Sahli, F. Al-Otibi, R.I. Alharbi, M. Amina, N.M. Al Musayeb, Sci Rep, **14**, 1 (2024)
6. Y. N. Slavin, H. Bach, Nanomaterials (Basel) **12**, 24 (2022)

7. G. E. Yılmaz, I. Göktürk, M. Ovezova, F. Yılmaz, S. Kılıç, A. Denizli, *Hygiene* **3**, 3 (2023)
8. S. Irum, N. Jabeen, K. S. Ahmad, *PLoS One* **15**, 12 (2020)
9. T. H. Phong, H. Tran, T. Hoang, M. Nguyễn, H. Khai, D. Cuong, V. Luan, N. Nam, N. D. Tan, *Plant Cell, Tissue and Organ Culture (PCTOC)* **155**, 1-13 (2023)
10. Y. Singh, K. Upendra, P. Sourav, B. Priyanka, M. Sheetal, S. Pooja, S. Vijeta, P.S. Krishna, C.W. Jason, P.D. Om, *Plant Physiol Biochem* **203** (2023)
11. M. Bakshi, A. Kumar, *Copper Nanostructures: Next-Generation of Agrochemicals for Sustainable Agroecosystems*. 393-413 (Elsevier, Egypt, 2022).
12. H. Chien, D. Nguyen, N. Huong, N. Le, K. Nguyen, H. Le, A. Nguyen, N. Dinh, S. Hoang, *J Plant Growth Regul* **41**, 364–375 (2022)
13. M. Priya, R. Venkatesan, S. Deepa, S.S. Sana, S. Arumugam, A.M. Karami, A.A. Vetcher, S.C. Kim, *Sci Rep* **13**(1) (2023)
14. S.E. El-Abeid, M.A. Mosa, M.A.M. El-Tabakh, A.M. Saleh, M.A. El-Khateeb, M.S. A. Haridy, *J Nanobiotechnology* **22**, 1 (2024)
15. J. Chen, L. Wu, K. Song, Y. Zhu, W. Ding, *J Integr Agric* **21**, 11 (2022)
16. O. Zakharova, E. Kolesnikova, E. Kolesnikov, N. Yevtushenko, V. Morkovin, A. Gusev, *IOP Conf. Ser: Earth Environ Sci* **595**, 1 (2020)
17. P. M. Evlakov, O. A. Fedorova, T. A. Grodetskaya, O. V. Zakharova, A. A. Gusev, Y. A. Krutyakov, O. Y. Baranov, *Nanotechnol Russ* **15**, 7 (2020): 476-82.
18. O. Fedorova, T. Grodetskaya, N. Evtushenko, P. Evlakov, A. Gusev, O. Zakharova, *IOP Conf. Ser: Earth Environ Sci* **875**, 1 (2021)
19. T. A. Grodetskaya, P. M. Evlakov, O. A. Fedorova, V. I. Mikhin, O. V. Zakharova, E. A. Kolesnikov, N. A. Evtushenko, A. A. Gusev, *Nanomaterials (Basel)* **12**, 5 (2022)
20. O. Zakharova, E. Kolesnikov, N. Shatrova, A. Gusev, *IOP Conf. Ser: Earth Environ Sci* **226**, 1 (2019)
21. M. Winkeljohn, V. C. Pence, *Appl Plant Sci* **10**, 5 (2022)
22. C. Walters, V. Pence, *Plants, People, Planet* **3** (2020)
23. A.T. Kramer, V. Pence, *Intl. Oaks* **23**, 91-108 (2012)
24. V. Chalupa, *Ann. For. Sci.* **50**, 295-307 (1993)
25. A.M. Vieitez, M. C. Sánchez, J. B. Amo-Marco, A. Ballester, *Plant Cell, Tissue Organ Cult* **37**, 287-95 (1994)
26. M. C. Sanchez, M. C. San-Jose, A. Ballester, A. M. Vieitez, *Tree Physiol* **16**, 8 (1996)
27. S. Mac An tSaoir, J O'Brien, *Irish Forestry* **56**, 2 (1999)
28. J. P. R. Martins, M. K. Wawrzyniak, E. M. Kalembe, J. M. Ley-López, J. M. S. Lira, *Plant Cell, Tissue Organ Cult* **156**, 1 (2023)
29. G. Lloyd, B. H. Mccown, *Commercially-Feasible Micropropagation of Mountain Laurel, Kalmia Latifolia, by Use of Shoot-Tip Culture* (1980)
30. B. Božilović, B. Nikolić, H. Waisi, J. Trifković, V. Dodevski, B. Janković, S. Krstić, M. Agronomy **13**, 7 (2023)
31. B. Kumar, M. Pal, P. Yadava, K. Kumar, S. Langyan, A. K. Jha, I. Singh, *PeerJ* **12** (2024)
32. J. Liu, L. Tu, M. Cheng, J. Feng, Y. Jin, *J Drug Deliv Sci Technol* **56** (2020)
33. M. Krstić, Đ. Medarević, J. Đuriš, S. Ibrić. *Lipid Nanocarriers for Drug Targeting*, (William Andrew Publishing, Norwich NY, 2018)

34. A. Śłosarczyk, I. Kłapiszewska, A. Parus, S. Balicki, K. Kornaus, B. Gapiński, M. Wieczorowski, K. A. Wilk, T. Jesionowski, L. Kłapiszewski, *Sci Rep* **13**, 1 (2023)
35. A. G. Kaningini, T. Motlhalamme, G. K. More, K. C. Mohale, M. Maaza, *Heliyon* **9**, 4 (2023)
36. A. Azam, A. S. Ahmed, M. Oves, M. S. Khan, A. Memic, *Int J Nanomedicine* **7**, (2012)
37. S. Banik, A. Pérez-de-Luque, *Span. J. Agric. Res* **15**, 2 (2017)
38. E. Ibarra-Laclette, J. Blaz, C. A. Pérez-Torres, E. Villafán, *J Fungi (Basel)* **8**, 4 (2022)
39. L. Dorjee, R. Gogoi, D. Kamil, R. Kumar, T. K. Mondal, S. Pattanayak, B. Gurung, *Front Microbiol* **14** (2023)
40. A. Varympopi, A. Dimopoulou, I. Theologidis, T. Karamanidou, A. Kaldeli Kerou, A. Vlachou, D. Karfaridis, *Pathogens* **9**, 12 (2020)
41. J.Chen, S. Mao, Z. Xu, W. Ding, *RSC Adv* **9**, 7 (2019)
42. J. Ramos-Zúñiga, N. Bruna, J. M. Pérez-Donoso, *Int J Mol Sci* **24**, 13 (2023)
43. I. H. Shah, M. A. Manzoor, I. A. Sabir, M. Ashraf, F. Liaquat, S. Gulzar, L. Chang, Y. Zhang, *Environ Sci Pollut Res Int* **30**, 18 (2023)
44. A. O. AlQuraidi, K. A. Mosa, K. Ramamoorthy, *Plants (Basel)* **8**, 1 (2019)
45. J. R. Velicogna, D. M. Schwertfeger, C. Beer, A. H. Jesmer, J. Kuo, H. Chen, R. P. Scroggins, J. I. Princz, *NanoImpact* **17** (2020)
46. L. Mahawar, M. Živčák, M. Barboricova, M. Kovár, A. Filaček, J. Ferencova, D. M. Vysoká, M. Brestič, *Plant Physiol Biochem* **206**, (2024)
47. G. Feigl, *J Plant Interact* **18**,1 (2023)
48. J. Zhang, X. Chen, Y. Song, Z. Gong, *J Integr Plant Biol* **66**, 3 (2024)
49. D. Arnaud, I. Hwang, *Mol Plant* **8**, 4 (2015)
50. D. Arnaud, S. Lee, *Plant Cell* **29**, 3 (2017)